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in Node-Negative Breast Cancer

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Although a majority of women with node-negative breast cancers have a good prognosis, 30% experience recurrence and death from metastatic disease. As result, systemic therapies are routinely administered to nearly all of these node-negative patients. Markers that better predict recurrence risk would more effectively target adjuvant therapies to the patients most likely to benefit from them. Our goal is to identify the genetic markers that 1) are differentially expressed in good versus bad outcome node-negative primary breast cancers, 2) help dichotomize node-negative patients into low and high-risk categories so that adjuvant treatment could be more effectively utilized, 3) identify genetic pathways associated with the metastatic phenotype. cDNA micro-arrays were used to analyze 30 untreated primary node-negative breast tumors from patients who were either completely cured by surgery alone (good outcome) and those who experienced metastatic recurrences (Bad outcome). At the $p=0.05$ level of significance, 137 genes involved in cell cycle, apoptosis, DNA repair, cell adhesion, cytoskeleton and signal transduction were found to be differentially expressed between the good versus bad outcome tumors by Wilcoxon tests.

Tree-view analyses generated dendrograms showing that the two categories of tumors mostly, but not completely, formed outcome-related clusters. We are currently validating the array data using semi-quantitative RT-PCR. Preliminary assays showed that the tested genes are indeed differentially expressed in the tumor samples. More candidate genes are currently being assayed. We have just started immunohistochemistry analysis on tissue arrays of archival specimens to assess the prognostic significance of some of these interesting candidate metastasis markers for which antibodies are commercially available.

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Introduction:

We compared gene expression profiles of untreated, node-negative, primary breast tumors from patients who either relapsed within 28 months (Bad outcome), or were cancer-free >10 years (Good outcome). The samples selected for this study were carefully matched for age, menopausal status, ER/PR status, tumor size and grade and other pathological parameters. Preliminary analysis was done by individual Wilcoxon tests for each gene across the arrays. Some of the microarray signal data that did not pass our normalization criteria were discarded from the study. The normalized data for the available samples was further subjected to clustering analysis using the Cluster/Tree View programs.

Some of the sorted genes from the clustering analysis are currently being validated by semi-quantitative RT-PCR. We are using rigorous and exhaustive criteria to validate the micro-array gene expression data. Firstly, the primer sequences are BLAST searched to ensure uniqueness. Secondly, the primers are tested on cDNAs generated from a mixture of cell line RNA and a trial set of tumor RNA to confirm a single product. Each primer pair is also optimized for the appropriate PCR cycles required for the gene amplification. An 18s rRNA control primer set is used as an endogenous control to normalize the target PCR product across the samples tested.

Key research accomplishments:

- 1) The preliminary data demonstrates that gene expression profiling of archived frozen tumors is indeed feasible and could distinguish heterogeneous tumor types based on gene expression differences. This signature patterns will be invaluable for clinicians to target adjuvant treatments only to those patients who are at-risk for recurrence.
- 2) Some of the genes could serve as novel bio-markers for metastatic recurrence, when further confirmed by Immunohistochemistry on Tumor Tissue Arrays. Further more, a few of the biologically relevant genes would be studied in detail for their role in metastatic pathways, in the final phase of this study.

Reportable Outcomes:

- 1) Clustering of the normalized gene expression data yielded better sorting of the genes, dichotomizing the two groups of tumors forming outcome based clusters.
- 2) Semi-Quantitative RT-PCR analyses is being used in validating the gene expression differences obtained from the micro arrays. We have thus far tested about 5 genes in an identical cohort of 3 of each good and bad outcome tumor RNA. More genes (15 more) are currently being assayed to validate their gene expression patterns and correlate with the micro-array data. As we await the completion of the validation assays, we expect to validate at least 50% of the selected genes based on initial assays (not shown).

3) A manuscript is currently under preparation based on this study.

Conclusions:

- 1) Cluster analyses proved to be a very useful tool to analyze complex expression array data that does not follow Normal distribution.
- 2) Wilcoxon analyses while useful to perform group comparisons, it obscures differences between heterogeneous tumor samples.
- 3) We have identified genes, some of which have been implicated in the metastasis of cancers. More recently, CyclinE has independently been shown to be a prognosticator in breast cancers, which has also panned out in our clustering data. We are currently evaluating other interesting genes as well. Once completed, the RT-PCR data will be correlated to micro-array data, to ascertain the up or down regulation of sorted genes.
- 4) We have also started the confirmation studies of the protein expression of some of these candidate markers on tumor tissue-arrays. Since not all proteins have commercially available and or reliable antibodies, we are currently in the process of developing immunohistochemistry (IHC) assays for the available antibodies on test tissue arrays.

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Appendices:

The cluster diagrams on the following pages demonstrate the quality of the profile data. Although, there is some interspersions of good and bad outcome cases, the majority of these tend to cluster together.

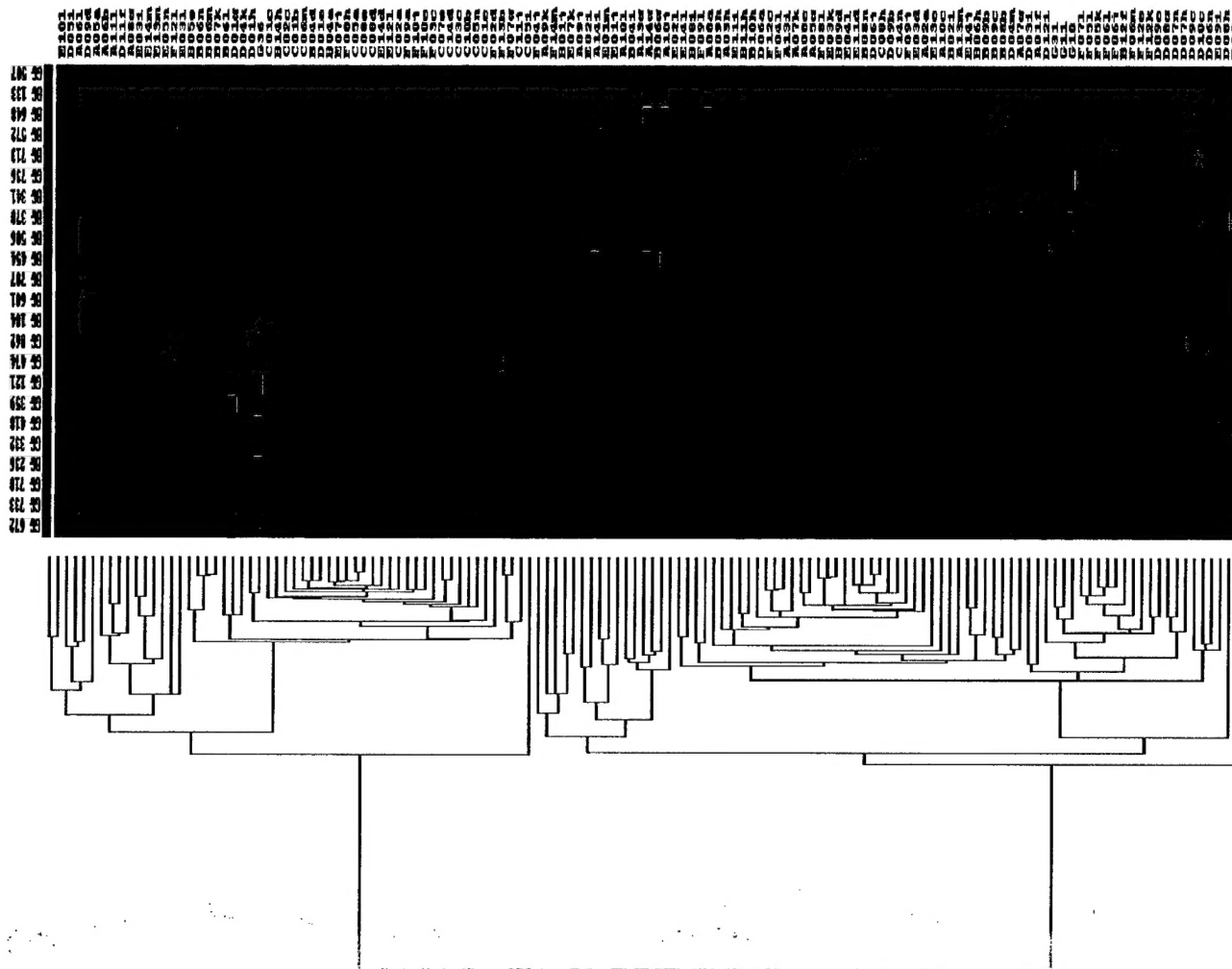


Figure 1: Clustering
of a set of 137 genes sel-
ected by Wilcoxon
analysis ($p=0.05$) show
visual differences bet-
ween the tumor types.

Figure 2: Cluster analysis generated dendrograms classifies the two types of tumors into outcome-related clusters. (scale: Dark Red-highest expression; Dark green lowest expression)

